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Karyotyping of *Cryptococcus neoformans* as an Epidemiological Tool

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Karyotyping of *Cryptococcus neoformans* var. *neoformans* can be used as an epidemiological tool for *C. neoformans* infections. In this study of over 40 isolates from both clinical and environmental sources, 90% had a unique chromosome banding by pulsed-field electrophoresis. There was no conserved pattern associated with body site of infection, geographical location of the isolate, or human immunodeficiency virus status. Karyotypes of individual isolates remained stable during both in vitro passage and in vivo infections. Karyotype was used to exclude the possibility of nosocomial spread of *C. neoformans* in one clinical situation and supported relapse in two other cases. Because of its variable sizes between isolates, karyotyping of *C. neoformans* is a convenient method for molecular identification of different strains.

Cryptococcus neoformans is an encapsulated pathogenic yeast which causes a significant number of infections in our enlarging immunocompromised populations. Fundamental questions regarding its epidemiology and pathobiology can now be answered by molecular biological techniques. For instance, the molecular cloning of genes and development of transformation systems have been performed so that basic mechanisms of virulence and gene regulations can be studied at the molecular level. On the other hand, basic studies on phylogeny and molecular epidemiology are also beginning. *C. neoformans* could be separated into four serotypes (a to d), but this scheme was generally not useful in the determination of clinical relatedness between strains. Recently, there have been several attempts to molecularly characterize *C. neoformans* so that different strains could be separated for epidemiological questions. Restriction fragment length polymorphisms of both mitochondrial DNA (20) and repetitive elements (16, 18, 22) or amplified ribosomal DNA (23) have been used to separate some strains. The random amplified polymorphic DNA technique of repetitive sequences is also being used successfully to separate different strains (9). In our opinion, another potentially useful and technically simple method for distinguishing different strains is molecular karyotyping using pulsed-field gel electrophoresis (15, 17).

Chromosome size variability has been a feature of some pathogenic yeasts, such as *Candida* species, with approximately seven to eight chromosomes (1, 6, 8), but may be less useful for other pathogenic fungi, such as *Coccidioides immitis*, with only three large chromosomes (12). In our original studies with *C. neoformans* karyotyping, we were impressed with the variability between various strains in chromosome sizes and yet with the stability of this feature for each strain passaged in vitro (15). On the other hand, the heterogeneity originally noted in clinical isolates was not as apparent when studies were performed to characterize *C. neoformans* var. *gatti*. In studies performed with environmental isolates from geographically separated areas, there were marked similarities among isolates in the size and number of their chromosomes (5). However, clinical isolates of *C. neoformans* var. *gatti* did show significant differences between strains. If this finding was also true for *C. neoformans* var. *neoformans*, the variety causing most clinical disease, then karyotyping may not be particularly useful for distinguishing different strains for epidemiological purposes. The findings with *C. neoformans* var. *gatti* karyotypes also suggest some concern with the possibility that infection itself causes shifts in chromosomal sizes, since clinical isolates showed karyotypic variability and environmental isolates showed karyotypic similarity. Also, regarding chromosome stability, in one strain of *C. neoformans* var. *neoformans*, it has been shown that selection for fluoro-orotic acid resistance produced karyotype instability (19). In this particular strain, even the process of transformation caused the appearance of a new minichromosome (21). In the following study, we report on the karyotyping of a variety of clinical and environmental isolates. We examined effects of in vivo growth and morphologic switching phenotype on karyotypes. Finally, karyotyping is used in several epidemiological circumstances to determine relatedness of strains.

MATERIALS AND METHODS

Isolates. Table 1 identifies several characteristics of each isolate. All were either clinical or environmental isolates of *C. neoformans*. H99 is a *C. neoformans* var. *neoformans* isolate used for years in our animal model (14), and its karyotype has been previously characterized (15). GBR is a morphologic switching clone of H99 which was produced by UV irradiation. It switches between white and pink colonies, is avirulent in the animal model, and is not a leaky adenine auxotroph.

Yeast DNA preparations. Three to four fresh colonies of each *C. neoformans* isolate from a yeast extract (1%)-peptone (1%)-glucose (2%) agar plate were inoculated into flasks with 50 ml of yeast nitrogen base (with 0.5% glucose) medium at 30°C in a shaking incubator for 24 h. A 1:300 dilution of these cells was made in 50 ml of fresh yeast nitrogen base and incubated for another 21 h. The cells were pelleted and suspended in 3 ml of 1 M sorbitol with 1 mg of lysing enzyme (Sigma, St. Louis, Mo.) per ml, and 30 μl of β-mercaptoethanol was added. The suspension was incubated for 1 h in a 37°C water bath. The cells were pelleted

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TABLE 1. Sources of *C. neoformans* isolates

Source	Isolate no.
Patients	
HIV infected	2, 4, 5, 8, 9, 11, 12, 15, 16, 19–21, 26, 29–34, 40
Tanzanian	43–46
HIV negative	1, 3, 6, 7, 10, 17, 18, 22–25, 27, 35–37
CSF	2, 4, 7, 9, 11, 12, 18, 23, 24, 30, 34–37, 39, 40, 43–46
Blood	3, 5, 6, 10, 19, 29, 31, 32
Environmental	13,[a] 14,[b] 28,[c] 38,[c] 41,[b] 42[d]

[a] From guano (Ohio).
[b] From pigeon (Ohio).
[c] From guano (North Carolina).
[d] From soil (Ohio).

and washed twice in 5 ml of SCE (1 M sorbitol, 0.1 M sodium citrate, 0.01 M EDTA [pH 9]). The cells were resuspended in 3 ml of SCE and added to 5 ml of 1% low-melting-point agarose in 0.125 M EDTA (pH 9) and poured into a 6-cm-diameter petri dish to solidify at room temperature. A 5-ml overlay (0.5 M EDTA [pH 9], 0.01 M Tris [pH 8], 1% sarcosyl, 1 mg of proteinase K) was added over the gel and incubated for 18 h at 50°C. The overlay was removed, and plugs were made. These plugs were stored in 0.5 M EDTA, pH 9.0, at 4°C until use.

Electrophoresis. Gel plugs containing *C. neoformans* DNA were inserted into a 1% agarose gel (4 by 4 in. [ca. 10 by 10 cm]). The DNA bands were separated in the gel by a CHEF DRII apparatus (Bio-Rad, Richmond, Calif.) in 0.5× Tris-borate-EDTA at 17°C. Electrophoretic conditions were as follows: for 16 h, 50- to 130-s periods at 125 V, and for 32 h, 170- to 300-s periods at 125 V. Gels were stained with ethidium bromide and photographed. With all electrophoretic gels, one lane contained the control H99 DNA for comparison of chromosomal sizes with those in other gels. H99 was used as the standard marker, and the size and stability of its chromosomes have previously been analyzed (15). Bands from the other strains were compared with the H99 chromosomal bands on each gel, and those bands with intensities of staining similar to those of the 1,650- and 1,494-kb chromosomes of H99 were considered multiple chromosomes and marked in heavy ink when recorded. All bands were charted on graph paper, assuming linear migration of the chromosomes, and are displayed in Fig. 1.

Animal model. A cryptococcal meningitis model in immunosuppressed rabbits was used to inoculate *C. neoformans* H99 (serotype a) intracisternally (14). At 4, 7, 11, and 14 days of infection, cerebrospinal fluid (CSF) was removed and cultured on Sabouraud agar plates. Two to three colonies were picked from each time point and grown in yeast nitrogen base broth for DNA preparation.

Clinical cases. Two cases of cryptococcal meningitis developed in 40- and 27-year-old males without evidence of underlying diseases, including human immunodeficiency vi-

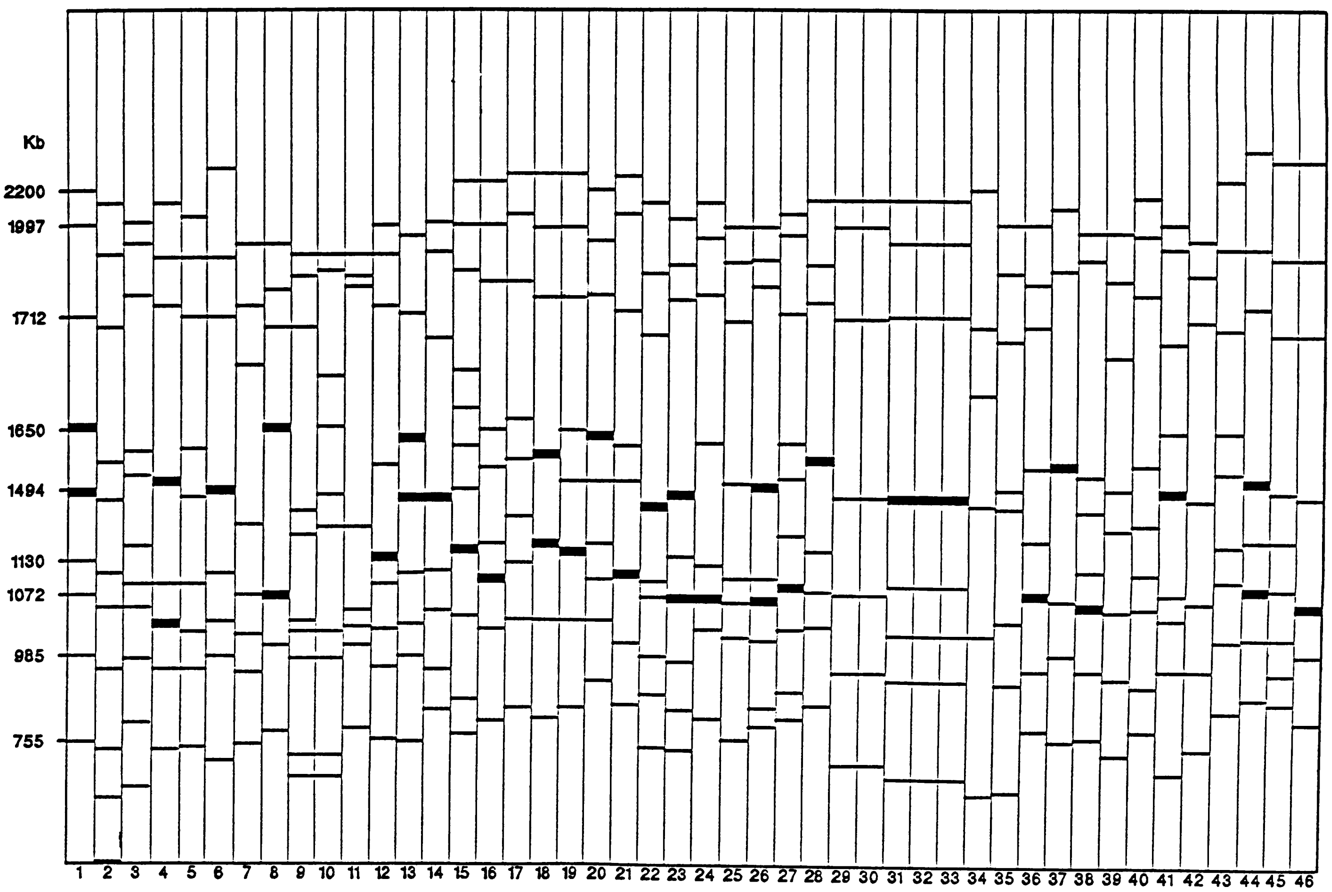

FIG. 1. Karyotypes of 46 clinical isolates of *C. neoformans*. On the ordinate are the sizes of the chromosomes from the standard *C. neoformans* strain, H99 (isolate 1). Heavy lines represent two or more chromosomes at the same location.

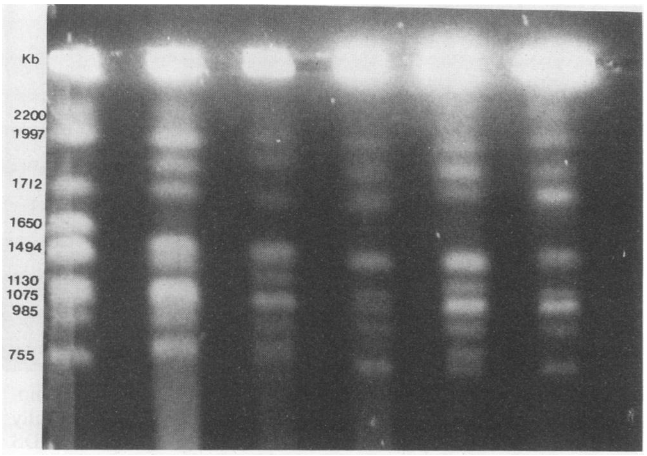

FIG. 2. Gel with ethidium bromide-stained chromosomal bands from six strains of *C. neoformans* (from left to right, isolates 1, 20, 21, 23, 22, and 24).

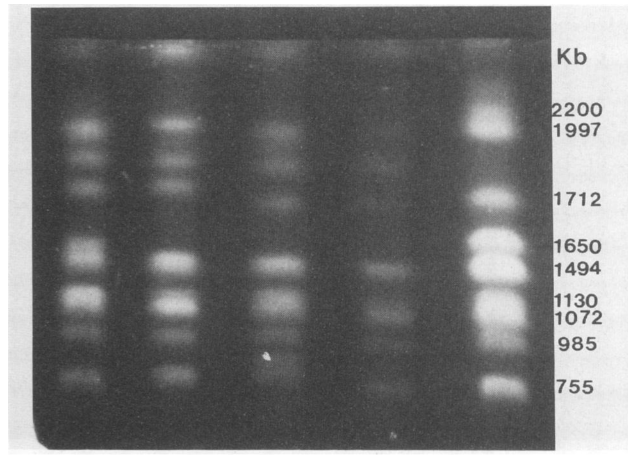

FIG. 3. Gel with ethidium bromide-stained chromosome bands from four African isolates and H99 (from left to right, isolates 43, 44, 45, 46, and 1).

rus (HIV) infection, approximately 2 months after ventriculoperitoneal shunt placement for subacute hydrocephalus by the same neurosurgeon on the same day (3).

One case of a clinical relapse of cryptococcal meningitis in a liver transplant recipient 1 to 2 months after stopping of amphotericin B (total dose, 1.5 g) was examined by comparing the original isolate with the relapse isolate, and a second case occurred in an AIDS patient who relapsed after receiving initial therapy with fluconazole for 6 weeks.

RESULTS

Table 1 describes the HIV status of hosts, African isolates, and the site of yeast isolation. There were 8 blood, 20 CSF, and 6 environmental isolates. The environmental isolates were found in soil or bird guano in North Carolina and Ohio, and two isolates were removed directly from the gastrointestinal tracts of two different pigeons in Ohio. The patterns of chromosome banding have been reconstructed in Fig. 1. Under the conditions set in this experiment, we identified a variable number of chromosome bands (from 7 to 12). Figure 2 represents a gel with karyotypes of six *C. neoformans* isolates, including H99. In all of the isolates tested, there were only two patterns found more than once. Despite multiple electrophoresis runs, there were three patient isolates which appeared to possess a conserved pattern (isolates 31 to 33) and two other patient isolates (isolates 29 and 30) which had similar karyotypes. There was no apparent geographical link among these patients, since their places of residence in North Carolina were hundreds of miles apart and hospital admission dates were not concurrent. By changing the length of the electrophoretic run and altering voltage of the contour-clamped homogeneous electric field, we might find differences in these karyotypes. Instead, we checked these five isolates by polymerase chain reaction fingerprinting using the core sequence of the wild-type m13 phage; they each appeared to have different amplified DNA patterns, which suggested different strains (9).

Although there were specific differences in some chromosome sizes for most strains, the karyotype patterns appeared to be similar to those in previous studies of *C. neoformans* (15). For instance, there were only a few isolates with chromosomes smaller than the 745-kb, or smallest, chromosome of H99, and the chromosome patterns were consistent with *C. neoformans* var. *neoformans* rather than the pattern of small chromosomes (less than 700 kb) seen frequently in *C. neoformans* var. *gatti* (15). This finding from karyotyping was supported by checking these isolates on CGB agar (10). All isolates in this study were identified as *C. neoformans* var. *neoformans* by this biochemical screening method. There were four clinical isolates from patients in Tanzania (African strains); these patients were infected with HIV. All isolates had different karyotypes, and there was no distinct pattern compared with those of the North American isolates (Fig. 3). There were also no specific pattern of chromosome banding in CSF or blood isolates and no conserved pattern in HIV-infected patients compared with patterns in non-HIV-infected patients. All the environmental isolates had different karyotypes (Fig. 4).

When H99 was inoculated into the subarachnoid space of

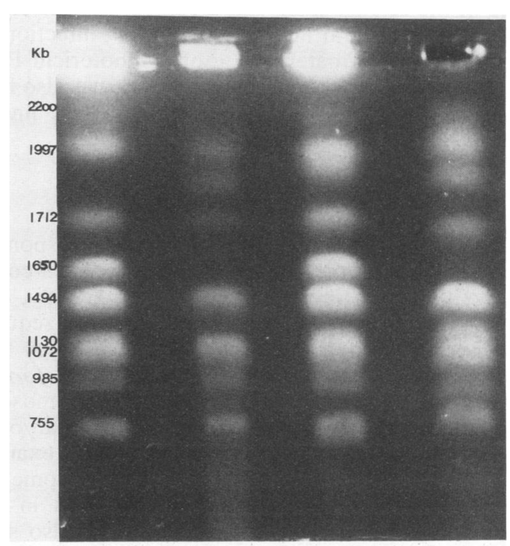

FIG. 4. Gel with ethidium bromide-stained chromosomal bands of three environmental isolates and the H99 standard (from left to right, isolates 1, 28, 13, and 14).

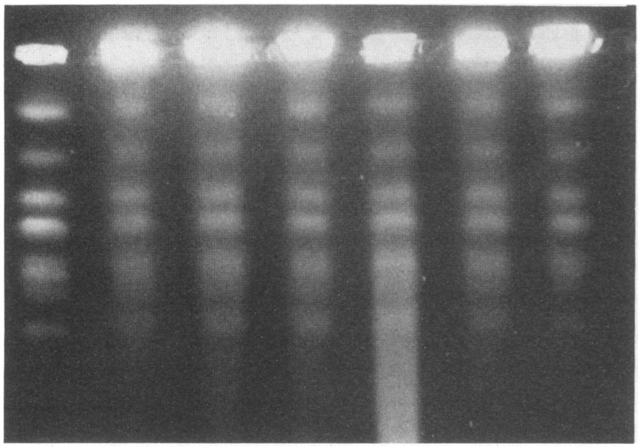

FIG. 5. Gel with ethidium bromide-stained chromosomal bands of H99 and colonies from this isolate during infection within the subarachnoid space (from left to right, H99, two colonies after 4 days of infection, two colonies after 11 days of infection, and two colonies after 14 days of infection).

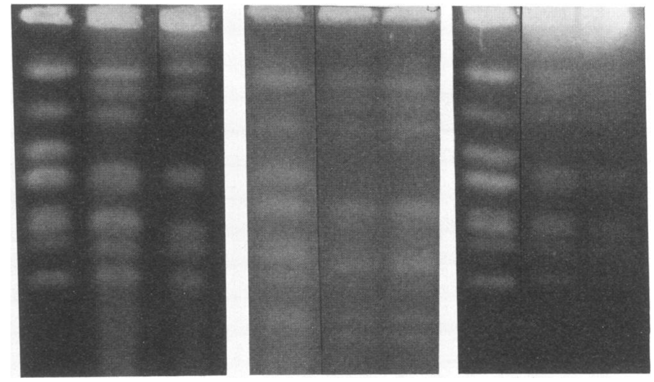

FIG. 6. Three gel sets with ethidium bromide-stained chromosomal bands. (Left to right) H99 and isolates from two temporally related cases (isolates 1, 36, and 37), H99 and isolates from an AIDS patient (isolate 1, initial isolate, and isolate after relapse), and H99 and isolates from a transplant patient (isolate 1, initial isolate, and isolate after relapse).

rabbits and multiple colonies were checked serially over a 2-week period of infection and compared with the original isolate, there was no detectable change in the karyotypes (Fig. 5). H99 has not changed its karyotype in over 5 years of in vitro passage, and a repeat chromosomal isolation of several other isolates did not change their karyotype in relation to the standard H99 karyotype. Since morphologic switching and the virulence phenotype have been associated with detectable chromosomal rearrangements in *Candida* species (4, 24), we checked a morphologically switching strain of *C. neoformans* (GBR), and this avirulent clone of H99 had the same karyotype as the parent.

In the clinical cases, we found the karyotyping useful for epidemiological purposes. In the two cases (cases 36 and 37) of cryptococcal meningitis which suggested the possibility of nosocomial transmission, the isolates possess significantly different karyotypes (Fig. 6) and thus were unlikely to be related or nosocomially spread. On the other hand, the karyotype of the relapse isolate in a clinical case of cryptococcal meningitis several months after initial infection and after receiving approximately 1.5 g of amphotericin B was identical to the original isolate, and similarity was also found in isolates from an AIDS patient (Fig. 6). These findings suggest true relapse rather than reinfection.

DISCUSSION

Although gene activities constantly change in response to environmental conditions, chromosomes have been considered relatively unchanging structures. Until recently, chromosomes were considered to be constant in both sequence and organization, and if there were changes they were produced by mobile elements (13). However, *C. neoformans* appears to have a polymorphic size of chromosomes between strains. There is precedent for this karyotypic variability in other eucaryotic pathogens. For example, *Plasmodium falciparum* has significant chromosome size polymorphism through deletions or duplications in both subtelomeric regions or repetitive regions, but also structural genes can be affected (2, 7). Also, the protozoan *Leishmania infantum* has shown significant chromosomal polymorphism, suggesting mechanisms of additions and deletions (11). We presently have no insight into the mechanism of chromosome variability in *C. neoformans* or its advantages for the yeast, but in one strain (3501, serotype D) with significant chromosome variability, transformation studies suggest very active telomerase activity through the known ability of the strain to place telomeres on introduced exogenous DNA and in one instance to actually produce a minichromosome (21).

As potentially interesting as karyotypic variation and its mechanism is for the biology of *C. neoformans*, our study attempted to determine whether it could be used for strain identification, with a particular emphasis on epidemiological studies. The results in the study affirm the use of this relatively simple technique for this purpose. Over 90% of the strains had unique karyotypes by using only one electrophoretic condition on a contour-clamped homogeneous electric field. In those with a conserved karyotype we did not find a direct link between patients but cannot rule out a common exposure. However, we also found by other methods (polymerase chain reaction fingerprinting) that it is probable that these isolates are truly different strains. Although these strains could not be resolved by the present karyotypic analysis, using a variety of electrophoretic conditions, resolution of differences might improve. Thus, further methods, including sequence information and fingerprinting, can potentially be used to confirm the relatedness of these strains when karyotype analysis is not conclusive. We found no conserved AIDS karyotype, a unique karyotype for environmental isolates, or any specific chromosomal patterns associated with the site of clinical isolation (CSF versus blood). On the other hand, both in the animal model and in patients with a relapse of infection, we did not detect an in vivo effect on individual strain karyotypes. Finally, in two cases which suggested possible nosocomial transmission we were able to detect significant differences in the strains' karyotype which suggest that these strains were different and not the result of surgical contamination.

It is clear that karyotyping of *C. neoformans* can be used for epidemiological studies such as determining environmental outbreaks or distinguishing between relapse and reinfection. Karyotyping could be used as the first screen, and for any strains with conserved karyotypes, further studies with other techniques, such as restriction fragment length polymorphism with repetitive elements, polymerase chain reaction fingerprinting, or specific sequence information, could further determine strain relatedness. Specific karyotype information may also be used in strain identification for genetic research purposes.

Finally, these results suggest that this yeast may have mechanisms which allow the dynamic alterations of chromosomes in nature, and we must be aware of the possibility of chromosome instability as work continues on the *C. neoformans* genome. The karyotype variability may be an important biological strategy for the fungus but presently can be used for epidemiological purposes.

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